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TYROSINE KINASE INHIBITION IN EXPERIMENTAL KIDNEY TRANSPLANTATION

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals.

I Rintala JM, Savikko J, Rintala SE, von Willebrand E. FK778 ameliorates post-transplant expression of fibrogenic growth factors and development of chronic rejection changes in rat kidney allografts. 2008. *Nephrol Dial Transplant.* 23(11):3446-55.

II Savikko J, Rintala JM, Rintala SE, Koskinen PK, von Willebrand E. Early short-term imatinib treatment is sufficient to prevent the development of chronic allograft nephropathy. 2011. *Nephrol Dial Transplant.* 26(9):3026-32.

III Rintala JM, Savikko J, Palin N, Rintala SE, Koskinen PK, von Willebrand E. Epidermal growth factor inhibition, a novel pathway to prevent chronic allograft injury. 2014. *Transplantation.* 27;98(8):821-7.

IV Rintala JM, Savikko J, Palin N, Rintala SE, Koskinen PK, von Willebrand E. Oral Platelet-Derived Growth Factor and Vascular Endothelial Growth Factor Inhibitor Sunitinib Prevents Chronic Allograft Injury in Experimental Kidney Transplantation Model. 2016. *Transplantation.* 100(1):103-10.

ABBREVIATIONS

APC	antigen-presenting cell
AZA	Azathioprine
CAD	chronic allograft dysfunction
CADI	Chronic Allograft Damage Index
CAI	Chronic allograft injury
CAN	chronic allograft nephropathy
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CsA	cyclosporine A
CTL	cytotoxic T lymphocyte
DA	Dark Agouti
DAMP	damage-associated molecular pattern
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ESRD	end-stage renal disease
bFGF	Basic fibroblast growth factor
GIST	gastro-intestinal stromal tumor
HLA	human leukocyte antigen
IFN-γ	interferon-gamma
IL-2	interleukin 2
IL-2R	interleukin 2 receptor
IMPDH	inosine 5-monophosphate dehydrogenase
iNOS	inducible nitric oxide synthase

MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
NK cell	natural killer cell
PAMP	pathogen-associated molecular patterns
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
ROS	reactive oxygen species
RT	room temperature
RTK	receptor tyrosine kinase
SMC	smooth muscle cell
SRL	sirolimus
Tac	tacrolimus
TCR	T-cell receptor
TGF-β	transforming growth factor beta
Th-cell	helper T-cell
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WF	Wistar Furth

INTRODUCTION

The development of modern surgery made experimental kidney transplantation possible at the beginning of the 20th century (Brown et al. 2010). In this early era, transplanted kidneys were rapidly lost due to non-surgical reasons that were impossible to investigate at the time. Increasing immunological knowledge led to the first successful human kidney transplant between identical twins in 1954 (Murray et al. 1958).

Initially, acute rejection was a major clinical problem, causing early graft losses, because the immunosuppressive drug regimen was narrow and ineffective (Calne 1968). Immunosuppressive treatment was revolutionized in the late 1970s when cyclosporine was introduced to treat acute rejection (Calne et al. 1978). Cyclosporine (CsA) treatment greatly improved transplantation results and made kidney transplantation a "gold standard" in the treatment of end-stage renal disease (ESRD). Cyclosporine was later followed by its competitor tacrolimus (Tac) (Kino et al. 1987), as well as other immunosuppressants, such as sirolimus (SRL) (Sacks 1999) and mycophenolate mofetil (MMF) (Platz et al. 1991). Combination therapies were developed to reduce the side effects of individual drugs and increase the immunosuppressive efficacy. These improvements further decreased the prevalence of acute rejection. However, long-term results failed to demonstrate significant progress in the prevalence of chronic rejection, and annual graft failure after 1 year has remained at 4% (Nankivell and Kuypers 2011).

Chronic rejection is a multifactorial process driven by both immunological and non-immunological factors, leading to organ failure (Nankivell and Kuypers 2011). ESRD alters the immunological response and may affect subsequent reactions to renal allografts (Anders et al. 2013). The transplantation process leads to both warm and

cold ischemia of the kidney graft and ischemia reperfusion injury after the graft is attached to the recipient's circulation. These events may affect activation of the recipient's immune system.

The immune system recognizes foreign tissue via multiple pathways. Innate immunity is the first-line, pattern-recognizing, defense system that reacts to foreign or injured tissue; it is activated after solid organ transplantation (Cucchiari et al. 2016). Adaptive immunity is a more sophisticated and pathogen-specific immunogenic process that leads to an alloimmune response targeting the transplanted kidney graft (Nankivell and Kuypers 2011). The alloimmune response causes acute rejection, which destroys the graft unless treated with immunosuppressive drugs. Although acute rejection may be treated successfully, it is a major reason for subsequent chronic rejection. Retrospective analysis has detected other risk factors for the development of chronic rejection, but detailed factors are still unknown. No treatment is currently available for chronic rejection.

Growth factors are ubiquitous peptides that mediate cell signaling and cellular processes in physiological and pathological environments. Many growth factors also participate in repair processes, such as wound healing (Bodnar 2015). Growth factors mediate their effects through specific receptors on the cell surface. Many of these receptors belong to the tyrosine kinase family, named for their induction of the intra-cellular phosphate binding of signaling molecules (Liu and Zhuang 2016). Growth factor expression is increased during both acute and chronic rejection (Savikko et al. 2001b) and participates in the interplay between innate and adaptive immunity (Kwan et al. 2014).

Pathological overexpression of growth factors has been shown to be a common feature in the development of various types of cancer (Odenthal et al. 2016). Interest

in the discovery of drugs that inhibit the effect of growth factors led to the development of imatinib, the first oral tyrosine kinase inhibitor, which inhibits platelet-derived growth factor (PDGF) signaling (Deininger et al. 1997). The discovery revolutionized the treatment of chronic myeloid leukemia (Druker et al. 2001). Other tyrosine kinase inhibitors have also been introduced for various cancers.

In transplantation studies, growth factors such as PDGF (Weinman et al. 2002), vascular endothelial growth factor (VEGF) (Jimenez-Sousa et al. 2012), epidermal growth factor (EGF) (Nakopoulou et al. 1994), and transforming growth factor beta (TGF-β) (Jimenez-Sousa et al. 2012) appear to be active in the rejection process, but their exact roles remain unknown. Standard immunosuppressive treatment fails to prevent the post-transplant growth factor induction (Bennett et al. 2016; McMorrow et al. 2005) that may be an important factor in the development of subsequent chronic rejection.

The aim of this study was to investigate the effect of four different growth factor response-inhibiting drugs on the development of chronic rejection and graft function in an experimental rat model of kidney transplantation.

REVIEW OF THE LITERATURE

1. CLINICAL KIDNEY TRANSPLANTATION

Despite the first experimental kidney transplantation being done in the beginning of the previous century, the first successful human kidney transplantation was not until 1954 (Murray et al. 1958). The procedure was performed between identical twins to avoid rejection. Since that time, kidney transplantation has been an option for treating ESRD. However, acute rejection was a major obstacle in the survival of renal grafts until the introduction of cyclosporine in the 1970s. Cyclosporine enabled dramatically better results and transformed kidney transplantation into the treatment of choice for ESRD patients (Calne et al. 1978). The most common reasons for ESRD and subsequent kidney transplantation are diabetic nephropathy, hypertension, chronic glomerulonephritis and cystic renal disease (Matas et al. 2015).

A shortage of kidney grafts limits the number of kidney transplantations, and 200-250 such procedures are performed in Finland annually. The outcomes of kidney transplantation are superior to those of dialysis treatment with regard to the mortality rate and quality of life (Czyzewski et al. 2014; van Walraven et al. 2010). In addition, kidney transplantation is highly cost-effective (Kalo et al. 2001, Ortiz et al. 2014).

The surgical results of kidney transplantation are good, though ESRD with prolonged uremia and vascular calcification often causes sclerotic arteries, which may complicate the creation of vascular anastomoses. Modern immunosuppressive medication has increased the successful treatment of acute rejection. With modern combination therapies biopsy proven acute rejection rate is 10-20% during the first 3 years after transplantation (Hanaway et al. 2011; Matas et al. 2015). However, acute

rejection remains the main reason for early graft loss within the first year after transplantation (El-Zoghby et al. 2009).

The development of modern immunosuppressive regimens has decreased graft loss due to acute rejection. However, the impact on long-term graft survival has been marginal (Matas et al. 2013; Nankivell and Kuypers 2011). Many different pathological causes contribute to late allograft loss (El-Zoghby et al. 2009), and the most prevalent cause of death among renal transplant patients is death due to cardiovascular diseases in the presence of a still functioning graft (Morath et al. 2007). However, immunologically driven chronic rejection remains a major reason for late allograft loss (El-Zoghby et al. 2009). No treatment is currently available for chronic rejection. Optimizing standard immunosuppression or changing to another regimen has been suggested as treatment, but the evidence of efficacy in chronic rejection is scarce (Pascual et al. 2002; Soliman et al. 2014) though improvements in graft function have been reported (Plischke et al. 2015). Instead, the prevention of metabolic syndrome, obesity, hypercholesterolemia, and hypertension (Hricik 2011) and smoking cessation (Opelz and Dohler 2016) have been suggested as possible interventions against late graft loss.

2. TRANSPLANT IMMUNOLOGY

The recognition of foreign or injured material is crucial for maintaining body physiology and homeostasis and has been secured with multiple pathways during evolution. The immune system recognizes foreign material by both non-specific innate immunity (Cucchiari et al. 2016) and antigen-specific adaptive immunity (Nankivell and Kuypers 2011). These processes partly overlap. After recognition, various signaling cascades aim to activate both cellular and humoral responses against

foreign material. Patient-derived conditions, such as age, sex, and comorbidities, may modulate the recipient's immune response. Clinically, these cascades lead to a rejection process.

2.1 DONOR TYPE

Most kidney allografts are derived from deceased donors. However, living kidney donation from a relative or non-relative is highly recommended to ease organ shortages and provide transplant options for ESRD patients. Living donor transplantations also result in lower graft failure rates than deceased donor grafts (Matas et al. 2013). Organs derived from deceased donors are typically retrieved from hospitals other than transplantation centers and have a longer cold ischemia time, which appears to activate innate immunity (Naesens et al. 2009). In addition, multi-organ procurement of deceased donors increases circulating inflammatory biomarkers that may reduce organ quality (Auraen et al. 2013). Furthermore, brain death itself activates an inflammatory cascade that may activate an immunological response and affect post-transplant events (Pullerits et al. 2015).

2.2 ISCHEMIA REPERFUSION INJURY

The transplantation procedure includes cold ischemia during the surgery and transportation of the graft. Cold ischemia causes hypoxic cell injury and ultimately leads to cell death if continuing for too long. Prolonged ischemia also leads to the accumulation of reactive oxygen species (ROS) (Aksu et al. 2015), which may cause additional tissue injury after the graft is anastomosed to the recipient's circulation (Cavaille-Coll et al. 2013). This process is called ischemia reperfusion injury. Ischemia reperfusion injury increases the post-transplant immunological response and

may be one factor in the initiation of the cascade from cell injury to increased inflammation, and even to acute and chronic rejection (Cavaille-Coll et al. 2013; Zhao et al. 2014).

2.3 INNATE IMMUNITY

Innate immunity is the first line of defense against pathogens and injured tissue. This process consists of natural killer (NK) cells, dendritic cells, monocyte-macrophages, neutrophils, and the complement system. Innate immunity is not allo-specific and lacks immunological memory. Instead, it is a pattern-recognizing response that reacts to any abnormal tissue residues encountered (Rosin and Okusa 2011). Therefore, it reacts to foreign tissue and injured tissue, inducing reparative cascades. Innate immunity is activated upon the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). In renal transplantation, innate immunity may react against DAMPs that are released during ischemia reperfusion injury (Cucchiari et al. 2016).

2.4 ADAPTIVE IMMUNITY

Adaptive immunity is a more specific immunological response that points to foreign antigens that are recognized by the immune system. After foreign tissue is recognized by antigen-presenting cells (APCs), they activate adaptive immunity through specific T-cells for cellular adaptive immunity and B-cells for humoral adaptive immunity. Adaptive immunity has long-term memory responses that enable rapid immune activation if the same pathogen or antigen is re-encountered in the future.

3. RENAL ALLOGRAFT REJECTION

Rejection is an immune response that induces and mediates injury in the allograft. Clinically, the rejection process is categorized as hyperacute, acute, or chronic. In addition to the rejection process, background pathology leading to the development of ESRD, such as diabetes or process behind glomerular disorder, may continue to affect the rejection process in a kidney graft.

Hyperacute rejection begins almost immediately after re-circulation of the graft and requires previous exposure to alloantigen. Therefore, this type of rejection can be prevented with adequate antibody cross-matching. Thus, hyperacute rejection is rare (Nankivell and Alexander 2010).

Acute rejection occurs in the early post-transplant period, most commonly in the first few months. Clinically, an increase in serum creatinine is considered a sign of acute rejection (KDIGO Clinical Practice Guideline 2009). Ultrasonography-assisted renal biopsies are taken when acute rejection is suspected, and episodes of acute rejection are treated with a short-term corticosteroid and by optimizing the immunosuppressive regimen (Baker et al. 2017).

Chronic rejection is a slower fibrotic process that leads to organ failure. It can begin any time after transplantation. Histologically, it is characterized by interstitial fibrosis, chronic inflammation, allograft arteriosclerosis, and tubular and glomerular lesions (Hayry et al. 1993; Solez et al. 1993).

3.1 ALLORECOGNITION

A key step in activation of the immune system in solid organ transplantation is the recognition of foreign tissue or material. The immune system recognizes foreign material based on cell surface antigens that differ from self-antigens (Nankivell and

Alexander 2010). All cell surfaces are coated with major histocompatibility complex (MHC) molecules that present cell antigens on the cell surface. MHC molecules are heterodimers consisting of α- and β-chains and form two classes: MHC I and MHC II. Human MHC molecules are called human leukocyte antigens (HLAs) (Nankivell and Alexander 2010). MHC I molecules are present in almost all nucleus-containing cells and present internal cellular peptides. MHC II molecules are found on the surface of APCs, such as macrophages, dendritic cells, and B-lymphocytes and present extracellular peptides/proteins (Caron et al. 2015).

3.2 ANTIGEN PRESENTATION

APCs, such as monocyte-macrophages and dendritic cells, are part of innate immunity and important cells in the initiation of the immune response. After these cells encounter foreign tissue, they internalize the foreign antigens and then present them to B- and T-cells to activate allo-specific adaptive immunity.

3.3 DENDRITIC CELLS

Dendritic cells are part of innate immunity. They recognize DAMPs by their pattern recognition receptors, of which the Toll-like receptors are the most investigated (Zhuang and Lakkis 2015). Dendritic cells are the most potent APC´s with regard to activating T-cells (Steinman and Idoyaga 2010) and present in the kidney, where their numbers increase during inflammation (Nelson et al. 2012). Recent findings suggest that recipient dendritic cells activate T-cells in the transplanted kidney and sustain the alloimmune response, indicating their profound role in the development of rejection (Zhuang et al. 2016).

3.4 MONOCYTE-MACROPHAGES

Monocytes are bone marrow-derived cells that enter organs after circulating for a few days in the bloodstream. In tissues, these monocytes differentiate into tissue macrophages and tissue dendritic cells (Rowshani and Vereyken 2012). Macrophages have no specific antigen receptors but are activated by cytokines, especially interferon-gamma (IFN-γ). Once activated, macrophages present two different states (Salehi and Reed 2015). M1 macrophages are mainly induced by IFN-γ and considered to be pro-inflammatory and cytotoxic, linked to the Th1 response. M2 macrophages are inducible by interleukin (IL)-4, IL-13, and TGF-β and associated with the Th2 response and tissue modeling (Salehi and Reed 2015). In addition, M2s secrete growth factors, such as TGF-β and PDGF (Rowshani and Vereyken 2012). Macrophages also secrete toxic molecules, including superoxides and nitric oxide via inducible nitric oxide synthase (iNOS), which function as pro-inflammatory cytokines, and tumor necrosis factor alpha (TNF-α), which may mediate the rejection cascade (Kutukculer et al. 1995; Salehi and Reed 2015).

3.5 NK CELLS

NK cells are part of innate immunity; therefore, they lack allo-specificity and a memory response. NK cells may modulate the allo-specific immunoresponse and activate T-cells (Zecher et al. 2012) but may also induce tolerance on islet allografts (Beilke et al. 2005). In addition, NK cells have been shown to aggravate graft injury (Hirohashi et al. 2012). NK cells recognize foreign material via the activation of their clono-typic antigen receptor and require co-stimulation through the balance between the stimulatory and inhibitory signals of different cell surface receptors (Beilke and

Gill 2007). The role of NK cells in solid organ transplantation remains controversial. However, NK cells may have a role in mediating long-term graft injury experimentally (Zhang et al. 2015).

3.6 ACTIVATION OF ADAPTIVE IMMUNITY

3.6.1 T-CELL ACTIVATION

T-cells mediate the adaptive immune response and are critical for the recognition of foreign tissue in a transplanted kidney graft in a process called allo-recognition. T-cells can be activated via three different cascades. In direct allo-recognition, T-cells recognize intact foreign material. In indirect allo-recognition, they recognize foreign antigens processed and presented by APCs. This process requires that recipient APCs have encountered foreign material, which they process and then present in their MHC II molecules. Recently, it was proposed that T-cells recognize intact foreign antibodies presented by APCs in a semi-direct manner (Ali et al. 2013).

After allo-recognition, T-cells are activated and further stimulate the immune response. However, full T-cell activation requires that T-cells bind MHC molecules via T-cell receptor (TCR), as well as co-stimulation. Otherwise, T-cells may become unresponsive in a process called anergy (Schietinger and Greenberg 2014). Various co-stimulatory routes have been described. The classical route includes B7 molecule on the APC surface and its binding to CD28 on the T-cell surface (Li et al. 2009).

T-cells are divided into CD4+ helper T (Th) cells, which activate other cell types, and CD8+ cytotoxic T-cells, which induce the apoptosis of foreign cells. In particular, CD4+ T-cells are important in initiating allo-recognition. When Th-cells become fully activated, they cause T-cell proliferation, clonal expansion, and cytokine expression

in an autocrine pathway. During activation, Th-cells may become Th1-, Th2-, or Th17-lymphocytes depending on the surrounding cytokine environment (Wood and Goto 2012). These different Th-subclasses have different responses. Th1-cells produce IFN-γ and IL-2, leading to CD8-cytotoxicity and macrophage activation (Abdoli and Najafian 2014). Th2-cells secrete IL-4, IL-5, and IL-13, which lead to antibody production and eosinophil activation, causing tissue destruction (Liu et al. 2013). Th17 can mediate glucocorticoid-resistant rejection (Nankivell and Alexander 2010). One of the key mediators in autocrine T-cell activation is IL-2. CD4+ Th-cells also activate B-lymphocytes, CD8+ T-cells and macrophages (Liu et al. 2013).

3.6.2 B-CELL ACTIVATION

B-cells recognize foreign antigens via their B-cell receptors, which bind to antigen. They also require co-stimulation via cell-cell interactions or surrounding cytokines (Stegall et al. 2014). Once fully activated, B-cells proliferate and differentiate into antibody-secreting plasma cells or memory B-cells (Kim et al. 2014). Allo-antibodies secreted by these plasma cells may cause graft injury in various ways via complement system and by activating cytotoxic cells (Kim et al. 2014).

3.7 REJECTION CLASSIFICATION AND SCORING

3.7.1 BANFF CLASSIFICATION

The Banff classification was created in the beginning of the 1990s to standardize the nomenclature and criteria for the diagnosis of rejection based on protocol biopsies (Solez et al. 1993). The Banff classification has been updated several times after its

original publication. In this classification system, renal biopsies are classified into six categories based on histopathological findings: normal, antibody-mediated changes, borderline changes, T-cell-mediated rejection, interstitial fibrosis, and tubular atrophy without specific etiology and changes not considered to be due to rejection. The Banff classification is routinely used in clinical transplantation to evaluate renal biopsies and originally introduced the term chronic allograft nephropathy (CAN), which was correlated with the term chronic allograft dysfunction or chronic rejection. CAN was introduced as a combination of several entities, including chronic rejection, calcineurin inhibitor (CNI) nephrotoxicity, and hypertensive changes. However, the term CAN was replaced in 2005 with the term "interstitial fibrosis and tubular atrophy without specific etiology" because CAN was considered to be used too liberally without attempting to specify the specific etiology (Solez et al. 2007). Therefore, aiming for a specific pathological diagnosis and terminology is currently recommended. However, this has led to somewhat arbitrary nomenclature and terms, such as chronic allograft injury (CAI) and chronic allograft dysfunction (CAD), which are used to describe multifactorial allograft dysfunction (Haas 2014).

3.7.2 CADI SCORE

The Chronic Allograft Damage Index (CADI) score is a sum of six parameters scored from 0-3, including interstitial inflammation and fibrosis, tubular atrophy, glomerular mesangial matrix increase, glomerular sclerosis, and arterial intimal proliferation (Isoniemi et al. 1992). The CADI criteria for inflammation and fibrosis are analogous with the Banff score so that inflammation scored 1, 2, and 3 with the CADI score is responding that of Banff score (interstitial fibrosis and total inflammation score in Banff 2007) (Solez et al. 2008). The CADI score is a numeric rejection score that

enables statistical comparisons between study groups. The score has been shown to predict the development of chronic rejection and outcome of kidney grafts in clinical kidney transplantation (Yilmaz et al. 2003).

4. IMMUNOSUPPRESSIVE MEDICATION

4.1 CORTICOSTEROIDS

Corticosteroids have been used in kidney transplantation since the beginning of the modern era. These drugs suppress cytokine expression in a non-specific manner and affect T-cell activation and macrophage function (Borrows et al. 2004). High doses of corticosteroids are required in solid organ transplantation, which causes difficult side effects such as secondary diabetes, obesity, hypertension, and a Cushingoid appearance. Corticosteroids are currently used mainly as induction therapy and periodically in cases of acute rejection. Otherwise, steroid-sparing therapy is recommended. Good clinical results have been obtained with steroid-sparing regimens including Tac and MMF (Borrows et al. 2004).

4.2 CALCINEURIN INHIBITORS

Cyclosporine was introduced in the late 1970s and revolutionized solid organ transplantation (Calne et al. 1978). Cyclosporine inhibits calcineurin, which is a critical enzyme in T-cell signaling. Without calcineurin, gene transcription of critical genes for T-cell activation, like IL-2, is suppressed (Camilleri et al. 2016). Cyclosporine has a narrow therapeutic window and multiple drug-drug interactions with other drugs because it effectively inhibits the CYP3A4 enzyme. Cyclosporine may cause nephro-, neuro-, and hepatotoxicity, affects lipid metabolism, and causes

hypertension (Gauthier and Helderman 2000) and hirsutism (Silva et al. 2014). In clinical transplantation, blood trough levels of cyclosporine are measured repeatedly to monitor the drug dose (Buchler et al. 2006).

Another CNI, Tac, was introduced in 1987 (Kino et al. 1987). Clinical trials showed that Tac is more effective in preventing acute rejection than cyclosporine (Pirsch et al. 1997). Tac also has a different side effect profile than cyclosporine, increasing the secondary diabetes rate compared to cyclosporine A (CsA), but it appears to have less cosmetic side effects (e.g., hirsutism) (Silva et al. 2014; Webster et al. 2005). In recent years, Tac has significantly increased its share as a primary CNI in renal transplant patients (Gardiner et al. 2016). Once-daily extended release Tac has increased patient compliance and reduced treatment failures because it has an easier dosage with a more consistent concentration profile (Bunnapradist et al. 2016).

4.3 ANTIPROLIFERATIVE DRUGS

Azathioprine (AZA) was widely used in kidney transplantation before CNIs were available. AZA is a non-specific antiproliferative drug that interferes with DNA synthesis and the function of various enzymes. AZA may cause side effects in rapidly proliferating tissues, such as bone marrow (Lennard et al. 1984).

MMF inhibits de novo purine synthesis by competitively inhibiting inosine 5-monophosphate dehydrogenase (IMPDH). Purine synthesis is needed in cell division (Platz et al. 1991). MMF suppresses T- and B-cell proliferation, clonal expansion, and the antigen-presenting capacity of dendritic cells. In addition, MMF decreases monocyte recruitment and, unlike CsA, is not fibrogenic (Allison and Eugui 2005). Instead, MMF appears to decrease graft fibrosis independent of its immunosuppressive mechanism (Mihovilovic et al. 2014). MMF also decreases acute

rejection (Ciancio et al. 2005) and graft loss (Knight et al. 2009; Wagner et al. 2015) compared to AZA in CNI combination regimens.

SRL was introduced in the 1970s and investigated as an antifungal drug, but it was abandoned due to side effects. Twenty years later it was discovered that SRL has structural similarities with Tac (Sacks 1999). SRL affects T-cell expansion downstream of IL-2R activation and inhibits the proliferation of both T- and B-cells. In addition, SRL inhibits responses to growth factors bFGF, PDGF, VEGF, and TGF-β (Morath et al. 2007). SRL has no nephrotoxic side effects but causes hyperlipidemia, anemia, edema, and an increased risk of cutaneous adverse effects (Woillard et al. 2012). SRL is currently being used in CNI-free immunosuppressive regimens that aim to prevent the nephrotoxic side effects of CNIs on kidney grafts. Everolimus (EVL) is an active metabolite of SRL (Morath et al. 2007).

4.4 ANTIBODIES

Specific immunosuppressive antibodies have been used in clinical transplantation to prevent acute rejection. Antibody therapy consists of repeated injections and may lead to anti-antibody production that decreases efficacy over time. Except for belatacept (see below), antibodies are mainly used in induction therapy and acute rejection episodes.

Polyclonal anti-thymocyte globulin is used mainly in high-risk patients (Wagner and Brennan 2012). Basiliximab is a chimeric (mouse/human) monoclonal antibody for IL-2R on activated T-cells. Alemtuzumab is a humanized anti-CD52 antibody for surface antigen CD52 expressed on T and B-cells, as well as NK cells and monocytes. Alemtuzumab may be more efficient than other antibodies in preventing acute rejection (Zhang et al. 2012).

Belatacept is a fusion protein of human IgG and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) that inhibits T-cell activation. It has been used in multidrug regimens instead of calcineurin inhibitors. Belatacept increases long-term patient and allograft survival, as well as graft function in clinical kidney transplantation compared to cyclosporine (Vincenti et al. 2016).

5. CYTOKINES AND GROWTH FACTORS

Cytokines are small soluble signaling proteins secreted by various cell types that act on other cells to change their function. These proteins mediate cell signaling and participate in various cellular adaptation processes, including immune and inflammatory responses (Dinarello 2007). Cytokines were originally named interleukins, chemokines, and lymphokines based, in part, on their discovery, but this nomenclature is somewhat arbitrary with regard to their function. Chemokines are small proteins that promote leukocyte migration into the allograft (O'Boyle, Ali et al. 2011) and are named after their activity as chemotactic cytokines. Interleukins mediate, in part, antigen presentation and the effector phases of allo-specific immune responses. Also growth factors are cytokines. Cytokines have few common features and many of their other attributes may even be contradictory. The cytokine environment defines the effect of cytokines on target cells and tissues (Dinarello 2007).

5.1 PLATELET-DERIVED GROWTH FACTOR (PDGF)

PDGF is a ubiquitous peptide that acts as a regulatory growth factor. It was originally discovered in the 1970s as a platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells (SMCs) in vitro (Ostendorf et al. 2014b;

Ross et al. 1974). Over-activation of PDGF is present in various tumor types, and its inhibition has been much studied in clinical oncology (Heldin 2013). Dimeric PDGF isoforms are composed of four different PDGF chains (A, B, C, and D). The A and B chains may unite to form three possible isoforms (PDGF-AA, PDGF-AB, PDGF-BB), whereas the PDGF-C and –D chains form homodimers (Ostendorf et al. 2014a). PDGF receptors (PDGFRs) consist of α and β subunits, which dimerize upon binding PDGF isoforms. PDGF-A and PDGF-C bind selectively to PDGFR-α, whereas the PDGF-B chain binds and dimerizes both PDGFR-α and PDGFR-β (Heldin et al. 2002). PDGF-D binds specifically to PDGFR-β (Bergsten et al. 2001). PDGF is a very powerful inducer of mesenchymal cell proliferation and its role in the proliferation of mesangial cells in kidney diseases has been well-characterized both in vitro and in vivo (Ostendorf et al. 2014a). PDGF-B possibly plays a central role in mediating glomerular mesangial cell proliferation and has been demonstrated to be a potent mitogen and chemoattractant for renal mesangial cells (Floege et al. 2008). PDGF also stimulates extracellular matrix production by proliferating renal interstitial fibroblasts (Lu et al. 2016; Tang et al. 1996). PDGF ligands and receptors are induced during acute renal allograft rejection (Savikko et al. 2001a). Both experimental and clinical studies have shown that PDGF induction is associated with chronic rejection (Alpers et al. 1996; Savikko et al. 2002). In addition, in an experimental rat model, PDGF inhibition almost completely prevents chronic allograft nephropathy (Savikko et al. 2003).

5.2 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF, previously known as vascular permeability factor, mediates both physiological and pathological angiogenesis and increases vascular permeability (Leung et al.

1989). In the early 1980s, VEGF was found to be a protein that induces vascular leakage (Ferrara 2009; Senger et al. 1983). Neo-angiogenesis enables tumor growth and VEGF plays a profound role in tumor neo-angiogenesis; therefore, its inhibition has been studied extensively in clinical oncology (Arjaans et al. 2016). Four different VEGF proteins (VEGF-A, -B, -C, and -D) have been described (Yamada et al. 1997). These proteins differ in their affinities to three VEGF receptors (VEFGRs); VEGFR-1, -2, and -3 are all RTKs (Claesson-Welsh 2016). In experimental kidney transplantation, VEGF expression is increased during both acute and chronic rejection (Malmstrom et al. 2008).

5.3 TRANSFORMING GROWTH FACTOR BETA (TGF-β)

TGF-β is present in serum, platelets, and macrophages and was found in the early 1980s to be an inducer of rapid fibroblast growth (Assoian et al. 1983). TGF-β plays a crucial role in the initiation, development, and metastasis promotion of various tumors. Its inhibition is an emerging intervention in clinical oncology, though it appears to have differential effects during different stages of carcinogenesis (Neuzillet et al. 2015). TGF-β activation has a key role in the fibrotic process of chronic kidney diseases (Vega et al. 2016). It induces cell proliferation in fibroblasts and causes mesenchymal cell matrix deposition through the promotion of extracellular matrix-associated gene expression and suppression of the activity of genes encoding matrix metalloproteinases, which degrade extracellular matrix (Border and Noble 1994; Leask and Abraham 2004; LeRoy et al. 1990; Liu et al. 2016). Serum TGF-β is mainly found in its inactive form associated with serum proteins and is activated by proteolytic cleavage (Annes et al. 2003). TGF-β in the renal interstitium is secreted by macrophages and causes the infiltration and activation of monocytes via monocyte

chemoattractant protein-1 (MCP-1) (Wang et al. 1999). TGF-β also causes the tubular epithelial to mesenchymal cell transition in which renal tubular cells transform into fibroblasts, and even further into myofibroblasts, which cause interstitial matrix accumulation and subsequent renal fibrinogenesis (Cho et al. 2016; Iwano and Neilson 2004). Elevated serum TGF-β concentration (Baczkowska et al. 2005) and renal tissue expression (Viklicky et al. 2003) has been shown to correlate with histological changes in chronic rejection. In addition, elevated urinary TGF-β concentrations in renal transplant recipients have been reported to correlate with proximal tubular injury (Teppo et al. 2004) and decreasing allograft function (Sibunruang et al. 2015).

5.4 EPIDERMAL GROWTH FACTOR (EGF)

EGF has various in vitro effects on both epithelial and mesenchymal cells; it promotes cell proliferation (Carpenter and Cohen 1976), chemotaxis (Blay and Brown 1985), and mitogenesis (Sherline and Mascardo 1982). EGF was found in the early 1960s as an inducer of mouse eyelid opening due to epidermal growth and keratinization (Cohen 1962). Over-expression of EGF is a common feature in various tumor types and its inhibition is used clinically to treat malignant diseases (Zeng and Harris 2014). EGF exerts its effects through its specific RTK and participates in multiple signal-transduction pathways. The protein also has various renal effects and may play a role in the development of diabetic nephropathy (Saad et al. 2005; Wassef et al. 2004). Furthermore, EGF decreases the glomerular filtration rate (Harris et al. 1988) and EGF receptor (EGFR) is an important mediator of renal endothelial dysfunction (Helle et al. 2009). According to microarray study of transplant biopsies, both EGF gene and protein expression is increased during allograft injury (Dosanjh et al. 2013)

In addition, inhibition of EGF has been shown to ameliorate experimental chronic renal fibrosis (Francois et al. 2004).

5.5 GROWTH FACTOR INTERACTIONS

Growth factors have wide range of interactions that modify their responses and effects (Figure 1). PDGF increases the sensitivity of fibroblasts to EGF activity (Wharton et al. 1983) and EGFR expression is critical for PDGF-induced fibroblast migration, suggesting a common downstream interaction (He et al. 2001; Li et al. 2000). Furthermore, PDGF mediates the interaction of TGF-β and EGF (Andrianifahanana et al. 2013). PDGF also induces VEGF (Hou et al. 2010), VEGFR (Edelberg et al. 1998; Stavri et al. 1995), TGF-β (Nishishita and Lin 2004), and bFGF (Sato et al. 1991), though PDGF-BB and bFGF interact directly, which appears to decrease their mitogenic activity (Russo et al. 2002). However, PDGF induces intercellular high molecular weight bFGF formation (Pintucci et al. 2005), which induces VEGF expression and mediates bFGF-VEGF interactions (Seghezzi et al. 1998). Furthermore, PDGF and bFGF have additive effect on chemotaxis (Ucuzian et al. 2010). EGF induces TGF-β expression (Qian et al. 2016; Van Obberghen-Schilling et al. 1988) and interacts with TGF-β in mediating the mitogenic effects on fibroblasts (Brinckerhoff 1983), renal fibrosis (Chung et al. 2013; Liu et al. 2015) and EMT (Docherty et al. 2006). EGF also induces VEGF (Frank et al. 1995; Kang et al. 2016) and has synergistic interactions with bFGF (Takeuchi et al. 1992). TGF-β induces EGFR expression (Assoian et al. 1984), as well as VEGF (Pertovaara et al. 1994; Suzuki et al. 2012) and bFGF (Finlay et al. 2000; Strutz et al. 2001) which also induces TGF-β. VEGF and FGF have synergistic effect in inducing PDGF (Kano et al. 2005).

Due to these interactions, growth factor expression may intensify after it is initiated and may be prolonged even after the initial cause has been excluded. These growth factor interactions are emphasized by the finding that growth factors may also intensify their own signaling cascades via autocrine loops, as shown with EGF (DeWitt et al. 2001; Earp et al. 1986), VEGF (Uchida et al. 1994; Vega-Diaz, Herron et al. 2001), bFGF (Miyamoto et al. 1998; Weich et al. 1991), TGF-β (Van Obberghen-Schilling et al. 1988), and PDGF (Eriksson et al. 1991; Sabuda-Widemann et al. 2009).

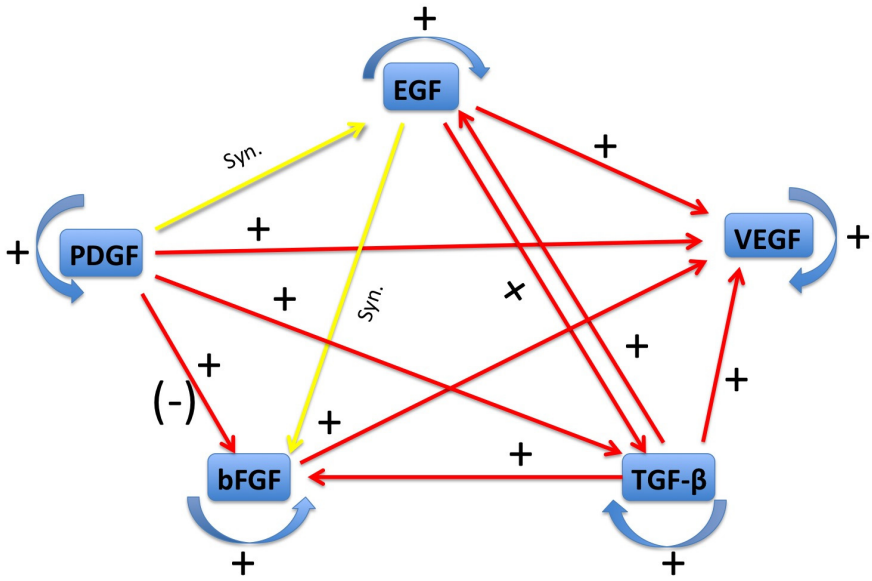

FIGURE 1: Growth factors have multiple interactions with other growth factors that may modulate the outcome of cytokine expression (red arrow and + represents inducing effect and yellow arrow and syn. represents syngenic effect. Blue arrow represents increased expression via autoregulation.)

5.6 GROWTH FACTORS IN END-STAGE RENAL DISEASE

PDGF expression is increased in diabetic nephropathy (Boor et al. 2014; Langham et al. 2003), and elevated urinary PDGF excretion correlates with the progression of nephropathy (Wang et al. 2009). In addition, VEGF (Bailey et al. 1999; Shao et al. 2016), TGF-β (Mou et al. 2016; Yamamoto et al. 1993), EGF (Chen et al. 2015; Gilbert et al. 1997), and bFGF (Vasko et al. 2009) are induced and active during diabetic nephropathy. Inhibition of PDGF (Lassila et al. 2005), VEGF (Sung et al. 2006), and TGF-β (Ziyadeh et al. 2000) has been shown to prevent histological changes in diabetic nephropathy, suggesting their causative role in the development of the disease.

In clinical glomerulonephritis, the expression of PDGF (Boor et al. 2014), TGF-β (Meng et al. 2014; Niemir et al. 1995), EGF (Bollee et al. 2011; Nakopoulou et al. 1994), and VEGF (Hohenstein et al. 2010) is increased. Evidence of increased bFGF expression and the benefits of bFGF inhibition in experimental glomerulonephritis have also been shown (Floege et al. 1998). In addition, inhibition of PDGF prevents experimental renal changes in glomerulonephritis (Iyoda et al. 2009).

The expression of PDGF (Liapis et al. 1997), VEGF, EGF, bFGF (Song et al. 2009), and TGF-β (Chea and Lee 2009) is increased in polycystic renal disease. In hypertensive nephrosclerosis, TGF-β is induced (Razzaque et al. 1996) and has been suggested to play a role in renal amyloidosis (Danilewicz and Wagrowska-Danilewicz 2006).

Diabetes is a major risk factor for cardiovascular diseases and diabetic nephropathy is one of the main indications for kidney transplantation. Inhibition of PDGF and VEGF has been shown to induce remission in diabetes (Templeton et al. 2008). In addition,

inhibition of PDGF appears to prevent both diabetic nephropathy and diabetes-associated atherosclerotic vascular lesions (Lassila et al. 2004; Lassila et al. 2005). Furthermore, inhibition of PDGF appears to limit the progression of chronic kidney disease in an experimental 5/6 nephrectomy model (Iyoda et al. 2011).

In summary, pathological growth factor expression is seen before and during the development of ESRD. This prolonged growth factor expression may affect subsequent development of rejection changes, as growth factor expression is already increased before kidney transplantation. Interestingly, growth factor inhibition has been shown to decrease the development of diseases that are primary reasons for ESRD and subsequent renal transplantation.

5.7 GROWTH FACTORS IN TRANSPLANTATION

ESRD patients are in a dual state of activated innate immunity, which may strengthen immune responses, and acquired immunosuppression, which may impair innate immunity (Anders et al. 2013). This dual state may affect the innate immune response in renal transplantation patients. Innate immunity has been shown to induce growth factor expression in patients with diabetes (Fischetti et al. 2011). VEGF also appears to activate NK cells (Cervi et al. 2007), and the inhibition of VEGFR blocks innate immunity-derived NK cell recruitment in animal models of cancer (Doloff and Waxman 2012). In addition, ischemia reperfusion injury induces growth factor expression (Spurgeon et al. 2005).

Growth factors also stimulate adaptive immunity and allo-recognition. PDGF and EGF stimulate T-cell proliferation and MHCII expression on APCs (Acres et al. 1985) and PDGF-inhibition decreases the differentiation of dendritic cells (Appel et al. 2004). T-cells express TGF-β, and this expression is significantly increased upon

T-cell activation (Kehrl et al. 1986). In addition, T-cell activation stimulates their VEGF expression, which is otherwise quiescent (Iijima et al. 1996). VEGF stimulates plasma cell proliferation (Rodriguez-Bayona et al. 2013). Macrophages secrete various growth factors, including PDGF, VEGF, TGF-β, and bFGF (Shimokado et al. 1985; Sunderkotter et al. 1994); their PDGF secretion is stimulated by IL-2 (Kovacs et al. 1994).

Human cytomegalovirus (CMV) infection is a frequent complication after kidney transplantation, with up to 26% of renal transplant patients infected during the first year after transplantation (Erdbrugger et al. 2015). CMV infection increases both graft loss and all cause mortality (Erdbrugger et al. 2015). EGFR plays an important role in both the mediation of viral entry into human monocytes and monocytic migration required for viral spreading (Chan et al. 2009).

Immunosuppressive drugs differ in their effects on growth factors. Cyclosporine has been shown to induce macrophage PDGF-B production in vitro (Iacopino et al. 1997). In addition, cyclosporine induces EGF (Chin et al. 2006) and TGF-β expression both in vitro and in vivo (Khanna et al. 1994, Shin et al. 1998), which may mediate the long-term nephrotoxic side effects of cyclosporine (Khanna et al. 1999). However, CsA appears to inhibit VEGF (Maruyama et al. 1992). Tac also induces TGF-β expression (Bennett et al. 2016; Khanna et al. 1999), but the in vitro effects of Tac have been investigated less than those of CsA. At least in hepatocytes, Tac has no effect on EGF and VEGF expression (Escribano et al. 2002; Serrano et al. 2006). SRL inhibits PDGF and bFGF-induced fibroblast proliferation in vitro (Salas-Prato et al. 1996) and is a known inhibitor of VEGF (Laugharne et al. 2007). However, SRL also induces TGF-β (Dodge et al. 2000).

According to experimental models, growth factor expression is induced in rejecting allografts (Savikko et al. 2001b) and, according to clinical studies, elevated serum VEGF levels predict the development of rejection in both cardiac (Aharinejad et al. 2009) and renal transplantation (Peng et al. 2008). Exogenous adenovirus-derived TGF-β (Csencsits et al. 2006), PDGF (Tuuminen et al. 2009), and VEGF (Lemstrom et al. 2002) induce chronic rejection in experimental cardiac transplantation models. Inhibition of TGF-β with anti-TGF-β antibody prevents chronic renal rejection (Guan et al. 2013). Furthermore, inhibition of PDGF has been shown to prevent chronic rejection in a rat model (Savikko et al. 2003). According to these results, growth factors are induced in rejecting allografts, cause rejection when used exogenously, and prevent rejection when inhibited. Therefore, growth factors appear to play a significant role in the development of the rejection process.

In summary, increased growth factor expression in solid organ transplantation is initiated by multiple pathways that are unaffected by immunosuppressive medications or induced by them. Early events such as ischemia reperfusion injury, innate immunity, allo-recognition, or T-cell activation may induce growth factors despite immunosuppressive medication. As growth factors induce their own expression, as well as the expression of other growth factors via interactions, this cascade may lead to prolonged growth factor expression and subsequent chronic rejection.

6. TYROSINE KINASE INHIBITION

6.1 TYROSINE KINASES AS GROWTH FACTOR RECEPTORS

RTKs are membrane-bound molecules that mediate growth factor signals from the cell surface to the intracellular space, leading to a growth factor response (Lemmon and Schlessinger 2010). In contrast, cytoplasmic tyrosine kinases mediate intracellular cascades. RTKs consist of an extracellular N-terminal ligand binding domain, a transmembrane domain, and intracellular C-terminal domain with tyrosine kinase activity. Upon ligand binding, RTKs undergo lateral dimerization in the membrane plane. These dimers have catalytic activity that leads to autophosphorylation of the RTK dimers in the intracellular domains. This phosphorylation then triggers signaling events (Hubbard and Miller 2007). In addition, RTKs have multiple additional phosphorylation sites for response modulation and interactions (Lemmon and Schlessinger 2010).

RTKs are structurally similar, and several subgroups exist, such as EGFRs, fibroblast growth factor receptors, insulin-like growth factor receptors, PDGFRs, and VEGFRs. Approximately 60 different RTKs are known, and many of them are growth factor receptors (Vlahovic and Crawford 2003).

6.2 TYROSINE KINASE INHIBITORS

Pathological growth factor expression is a common feature in various cancers and a possible point of intervention. Therefore, vast resources have been used to develop growth factor-inhibiting drugs. Imatinib was the first oral TKI that revolutionized the treatment of chronic myeloid leukemia (Druker et al. 2001). Several other TKIs that inhibit different RTKs have followed (Broekman et al. 2011). TKIs are administered

orally once or twice daily, and their effect is achieved by competitive inhibition of intracellular ATP binding on the RTK. TKIs differ in their selectivity. Some TKIs are called pan-TKIs or multi-target TKIs because they inhibit several RTKs, whereas others are more selective (Broekman et al. 2011).

TKIs inhibit several signaling cascades and, especially pan-TKIs, may interfere with physiological cell signaling. However, TKIs are considered to be well tolerated in clinical oncology. In the field of transplantation, the acceptable level of adverse effects is certainly lower than in the treatment of malignancies. However, tyrosine kinases are increasingly being investigated outside oncology and are considered for use even in renal autoimmune diseases (Wallace and Gewin 2013). Therefore, they could be considered in transplantation regimens.

6.2.1 FK778

Leflunomide (LFM) is used as an immunosuppressant in rheumatoid arthritis. It has potent immune-modulatory and anti-inflammatory effects but a long half-life that has restricted its use as an immunosuppressant. FK778 is an analogue of the active metabolite of LFM. With a shorter half-life than LFM, FK778 could be a more useful choice in solid organ transplantation (Fitzsimmons and First 2004; Vanrenterghem et al. 2004). Similar to LFM, FK778 inhibits T- and B-cell proliferation and interferes in de novo pyrimidine biosynthesis by inhibiting the dihydro-orotate dehydrogenase (Fitzsimmons and First 2004). Additional immunosuppressive effects of LFM and FK778 are achieved by the inhibition of several growth factor RTKs, such as EGF and VEGF, which have been shown to be inhibited by LFM in vitro (Bartlett et al. 1991; Shawver et al. 1997). LFM also inhibits PDGF RTK activity and strongly inhibits the growth of PDGFR-overexpressing glioma in vivo (Xu et al. 1999). FK778

decreases neointimal proliferation and is thus vasculoprotective independent from dihydro-orotate dehydrogenase inhibition (Savikko et al. 2003). FK778 also reduces endothelial adhesion molecule upregulation and attenuates lymphocyte-endothelium interactions both in vitro and in vivo (Deuse et al. 2004; Schrepfer et al. 2005).

6.2.2 IMATINIB

Imatinib, an orally administered RTK inhibitor, is well known as the first small molecule inhibitor of tyrosine kinases to be licensed for cancer treatment. Imatinib inhibits PDGF RTK (Buchdunger et al. 2000). Imatinib has been shown to inhibit both PDGF-α and -β receptors (Buchdunger et al. 2000). It has also been demonstrated to inhibit TGF-β signaling via a non-Smad TGF-β pathway (Wang et al. 2005).

In clinical oncology, imatinib has been shown to be well-tolerated and effective in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumor (Joensuu et al. 2013; Yeung et al. 2015). The most frequent adverse effects of imatinib are fluid retention, abdominal disorders, skin rash, and hematological disorders (anemia, neutropenia, and thrombocytopenia). In long-term oncological usage, only 21.8% of patients have grade 3-4 adverse effects (National Cancer Institute common toxicity criteria, indicating severe or life-threatining adverse effect) (Kalmanti et al. 2015).

In addition to approved indications, trials have suggested that imatinib has efficacy against desmoid tumors (Chugh et al. 2010), pulmonary manifestations of systemic sclerosis (Fraticelli et al. 2014), and recurrent dermatofibrosarcoma (Ugurel et al. 2014). A combination of imatinib with traditional chemotherapy may offer antitumor activity in metastatic solid tumors, such as breast cancer and soft-tissue sarcomas (Pishvaian et al. 2012), as well as advanced endocrine tumors (Halperin et al. 2014).

Interestingly, imatinib has efficacy against steroid-refractory graft versus host disease after stem cell transplantation (Olivieri et al. 2013).

Case studies have suggested that imatinib may be effective against Crohn´s disease (Magro and Costa 2006) and rheumatoid arthritis (Pereira et al. 2010). Based on various immunomodulatory findings, imatinib has been suggested as a treatment for immune-mediated kidney injury (Wallace and Gewin 2013).

Experimental data suggest that imatinib may have a beneficial effect against neurobehavioral deterioration after subarachnoid hemorrhage (Shiba et al. 2013) and multiple sclerosis (Adzemovic et al. 2013). In addition, imatinib prolongs the time-frame for thrombolytic treatment after stroke (Su et al. 2008) and attenuates experimental forms of diabetic nephropathy (Lassila et al. 2005) and diabetic atherosclerosis (Lassila et al. 2004).

6.2.3 ERLOTINIB

Erlotinib is a highly selective, novel oral TKI for EGFR (Pollack et al. 1999). The concentration required to inhibit PDGF is >1000-fold that needed for EGF inhibition (Pollack et al. 1999). Erlotinib is clinically used for non-small cell lung cancer and pancreatic cancer, and it prolongs progression-free survival in both of these malignancies (Cappuzzo et al. 2010, Moore et al. 2007). Based on clinical trials, erlotinib is well tolerated, and only 16% of patients required a dose reduction; the main adverse effects were skin rash and diarrhea (Neal 2010). In addition, preliminary reports indicated that erlotinib may have efficacy against myelodysplastic syndrome (Thepot et al. 2014), as well as against head and neck cancer (Rosenthal et al. 2014). Experimentally, erlotinib inhibits the development of dysplastic lesions in colon (Pagan et al. 2011).

6.2.4 SUNITINIB

Sunitinib is a TKI for gastrointestinal stromal tumor (GIST) and metastatic renal cell carcinoma (Chow and Eckhardt 2007). It inhibits PDGFR-α and –β and VEGFR-1, -2, and -3 at similar concentrations (Mendel et al. 2003). Sunitinib has been used successfully in the treatment of imatinib-resistant tumors, which indicates better efficacy, although the effect is at least partly dependent on the genetic factors of the tumor (Heinrich et al. 2008). However, sunitinib is more effective than imatinib in preventing PDGFR-β phosphorylation (Abrams et al. 2003). Efficacy against differentiated thyroid cancer (Bikas et al. 2016), advanced melanoma (Decoster et al. 2015), recurrent endometrial carcinoma (Castonguay et al. 2014), and aggressive fibromatosis has also been reported (Jo et al. 2014).

Sunitinib appears to increase glucose control in type 2 diabetes patients (Oh et al. 2012) and, according to a case study, even individual type 1 diabetes patients may benefit from sunitinib (Huda et al. 2014). Interestingly, sunitinib may interfere with innate immunity via reductions in cytokine levels (Jha et al. 2011).

Sunitinib inhibits various tyrosine kinases and has a wider range of adverse effects than imatinib or erlotinib. The most frequent side effects are diarrhea, fatigue, and nausea. Hypertension, hand and foot syndrome (dysesthesia and tingling of the palms, fingers and soles of the feet), and hematological adverse effects are also rather common. Fifty-two percent of patients have grade 3 or 4 adverse effects during the first year of sunitinib treatment, but after that the adverse effects steadily decrease, except for hypothyroidism which had a cumulative incidence of 36% over 6 years (Porta et al. 2016).

AIMS OF THE STUDY

The aim of this study was to investigate different TKIs and their effect on growth factor expression and the development of acute and chronic rejection in an experimental rat kidney transplantation model.

The specific aims of this study were:

1. To investigate the effect of FK778 on acute and chronic rejection in both monotherapy and combination therapy with cyclosporine and tacrolimus.
2. To investigate the effect of FK778 on post-transplant growth factor expression.
3. To investigate whether short-term imatinib treatment is sufficient to prevent later chronic rejection changes in kidney grafts.
4. To investigate the effect of EGF inhibition with erlotinib on acute rejection and the development of chronic rejection.
5. To investigate the effect of combined inhibition of PDGF and VEGF with sunitinib on the development of chronic rejection.

METHODS

1. EXPERIMENTAL STRATEGY

Our aim was to investigate the effect of various human drugs in an experimental kidney transplantation model of chronic rejection. Because acute rejection is a major reason for the development of chronic rejection, we used a 5-day transplantation model to investigate early events and acute rejection and a 90-day transplantation model to investigate chronic rejection. Right-side nephrectomy was performed during the primary operation and a left-side nephrectomy after week 1. From weeks 2 to 12. the chronic study groups were dependent on the transplanted kidney graft for their renal function. During the longer study period, we obtained weekly blood samples to measure serum creatinine as an indication of graft function.

Many of the drugs used in our experiments were originally tested by their developers in mouse models, and ready-to-use doses for rat studies could not be taken from the literature. Therefore, we performed dose-response studies with an experimental rat aorta denudation model (III and IV) to define drug doses that could be used in a more demanding and time-consuming transplantation model. The denudation model also mimics transplant vasculopathy, offering new knowledge on the effects of these drugs. Because cellular pathways that mediate the effect of TKIs on the rejection process are somewhat unclear, we also performed vitro studies to investigate the effect of sunitinib on SMCs (IV). Histological results were analyzed by the CADI score and immunohistochemistry was used to define growth factor expression in kidney grafts (I-IV).

2. EXPERIMENTAL ANIMALS

Male Wistar rats (Harlan, the Netherlands) weighing 350 to 370 g were used for aorta denudation. Specific, pathogen-free, inbred male Wistar Furth (WF; $RT1^u$) and Dark Agouti (DA; $RT1^a$) rats (Harlan, the Netherlands) weighing 300–350 g were used for renal transplantation. These inbred rat strains have a well-defined major MHC-mismatch that results a severe mixed interstitial-vascular type acute rejection with tubular and glomerular necrosis by day 7 unless treated with immunosuppressive regimen (Savikko et al. 2001a). All animal surgeries were performed using isoflurane (Baxter, Deerfield, IL) induced anesthesia. The rats received regular rat food and tap water ad libitum and were maintained on a 12-h light/dark cycle. Buprenorphine (Temgesic; Reckitt & Colman, Hull, England) was used for postoperative pain relief. The animals received human care in compliance with the Guide for the Care and Use of Laboratory Animal Resources published by the National Institutes of Health and Office of Animal Care and Use (National Research Council, Washington, DC, National Academy Press 1996).

3. RAT AORTA DENUDATION MODEL

The aortas were denuded of endothelium by intraluminal passage of a size 2 French Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Santa Ana, CA) introduced through the left iliac artery during isoflurane-induced anesthesia. The rats were orally treated with erlotinib (1, 5, or 10 mg/kg/d; Roche, Basel, Switzerland), sunitinib (1, 5 or 20 mg/kg/d; Phizer, New York, NY) or vehicle only (polyethylene glycol) once a day. Aortic samples were harvested 14 days after injury and histological changes and the size of the neointimal area evaluated from midaortic

sections. The neointimal area was digitally analyzed using Axioplan 4.2 analysis software (Carl Zeiss, Oberkochen, Germany).

4. EXPERIMENTAL KIDNEY TRANSPLANTATION IN RATS

Transplantations were performed from DA to WF rats using a modified microsurgical technique (Savikko, Kallio et al. 2001a, Fisher and Lee 1965). Syngenic transplantations from DA to DA rats were used as controls. The donor kidney was transplanted heterotopically to the recipient´s abdominal aorta and inferior vena cava using an end-to-side aortic and vena caval anastomosis. Uretral anastomosis was performed end-to-end close to the renal pelvis. The right native kidney was removed during transplantation and a left nephrectomy performed postoperative day 7 for animals in chronic rejection groups. In the acute rejection groups, the left kidney was left in situ because the animals were allowed to recover from the primary operation. The total perioperative ischemia time was standardized between 50 and 60 min, beginning with exsanguination and cold perfusion of the graft and ending with revascularization of the graft.

Rats were medicated daily via lavage (per orally) or needle (subcutaneusly) by researchers and their overall condition and behavior checked during the medication process. In addition, all study animals were checked daily by independent animal specialists to monitor their well-being.

5. DRUG REGIMENS

After transplantation, the rats were medicated once daily. Buprenorphinum (Temgesic; Reckitt & Colman, Hull, England) was used for postoperative pain relief after transplantation and after nephrectomy in the chronic groups.

CsA (Sandimmun, Novartis, Basel, Switzerland) was purchased from the local hospital pharmacy, dissolved in intralipid (Kabi Vitrum, Stockholm, Sweden) to a final concentration of 1.5 mg/ml, and administrated subcutaneously. Tac (kindly provided by Fujisawa, Osaka, Japan) was dissolved in distilled water to a final concentration of 1.5 mg/ml and administrated orally. FK778 (kindly provided by Fujisawa) was dissolved in 1% carboxymethyl cellulose to a final concentration of 5 or 10 mg/ml and administrated orally. Imatinib (Glivec, Novartis) was purchased from the local hospital pharmacy, dissolved in water to a final concentration of 10 mg/ml, and administered orally once a day.

Erlotinib (kindly provided by Roche) was dissolved in polyethylene glycol (Sigma-Aldrich, St. Louis, MO) and administered orally daily at 10 mg/kg/d. Sunitinib (kindly provided by Pfizer) was dissolved in polyethylene glycol (Sigma-Aldrich) to a final concentration of 2 mg/ml.

For acute rejection experiments, rats were treated with a monotherapy of CsA (1.5 mg/kg/d), Tac (1.5 mg/kg/d) or FK778 (5, 10 or 20mg/kg/d, I) or with a combination-therapy of CNI (CsA or Tac) and FK778 (10mg/kg/d, I), erlotinib (10mg/kg/d, II) or sunitinib (2mg/kg/d, IV). In chronic rejection experiments CNI (CsA or Tac) was used as a monotherapy or combined with FK778 (10mg/kg/d, I), imatinib (10mg/kg/d, II), erlotinib (10mg/kg/d, III) or sunitinib (2mg/kg/d, IV).

6. HISTOLOGY

Histological staining was done to enable histological evaluation of rejection changes using the CADI score.

The kidney grafts were bisected horizontally and fixed in 4% paraformaldehyde. The specimens were cut into 2-µm-thick sections and stained with Mayer's hematoxylin-

eosin (HE), Masson's trichrome, and diastase-periodic acid-Schiff (D-PAS) to investigate chronic rejection changes.

Histopathological analysis was done in a blind review. To investigate acute rejection, the intensity of perivascular and diffuse interstitial inflammation was scored in the samples as follows: 0, no inflammation; 1, faint inflammation; 2, moderate inflammation; and 3, intense inflammation. Chronic rejection changes were scored according to the CADI, which consists of six parameters scored from 0-3, including interstitial inflammation and fibrosis, tubular atrophy, glomerular mesangial matrix increase, glomerular sclerosis, and arterial intimal proliferation. The CADI grading of fibrosis and tubular atrophy from 1-3 is analogous to the Banff score (Banff 2009, category 5 grades I, II, and III) (Sis et al. 2010). The CADI score is a numeric rejection score that enables statistical comparisons between study groups.

7. IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was used to define post-transplant growth factor expression and localization. The samples were incubated in 4% paraformaldehyde for 4 h and then routinely fixed in paraffin blocks. Sections (4-µm-thick) were cut in series on glass slides. Before staining, the samples were deparaffinized. For epitope retrieval, the glass slides were heated in a microwave oven for 20 min in sodium citrate buffer (pH 6.0) and then allowed to cool to room temperature for 20 min. To demonstrate the expression and localization of growth factors, the samples were immunostained using the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA) or the EnVision™ G|2 System/AP, Rabbit/Mouse (Permanent Red) kit (Dako Systems, Glostrup, Denmark) according to the manufacturer´s instructions. The final staining reaction was revealed by NovaRed (Vector Laboratories) according

to the manufacturer´s instructions, yielding a red reaction product. After staining, the slides were counterstained with Mayer's hemalum and permanently mounted after incubation in alcohol series and xylene. Negative controls without primary antibody were used for all staining experiments.

Polyclonal goat IgG antibody to PDGF-A (2 µg/ml, sc-128, Santa Cruz Biotechnology, Inc., CA), PDGF-B (5 µg/ml Abcam, Cambridge, UK), PDGFR-α (10 µg/ml, Lab Vision Corp, Fremont, CA), PDGFR-β (2 µg/ml, Santa Cruz), VEGF-A (2 µg/ml; Santa Cruz), VEGF-B (2 µg/ml; Santa Cruz), VEGFR-1 (2 µg/ml; Santa Cruz), VEGFR-2 (2 µg/ml; Santa Cruz), TGF-β (2 µg/ml, Santa Cruz), TGF-βR1 (2 µg/ml, Santa Cruz), EGF (200 µg/ml; sc-1343; Santa Cruz), and EGF-R (200 µg/ml; sc-03, Santa Cruz) were purchased from commercial suppliers.

Immunohistochemical analysis was done in a blind review. The intensity of the staining was scored in the samples as follows: 0, no visible staining; 1, cells with faint staining; 2, moderate intensity with multifocal staining; and 3, intense diffuse staining.

8. IN VITRO STUDIES

In vitro studies of SMC proliferation were performed to investigate the pathway that could mediate the effect of TKIs on the histological rejection process. Rat coronary artery SMCs (kindly provided by Professor Dariusz Leszczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 1% glutamine (Invitrogen), and 2.5% penicillin and streptomycin (Invitrogen). For the proliferation study, 500 cells were cultured in 96-well culture plates for 24 h following 72 h in serum-free media. After serum starvation, the cells were stimulated to proliferate with

2 ng/ml of rat recombinant PDGF-BB (Sigma-Aldrich, St. Louis, MO), a known SMC inductor (Nykanen et al. 2005). Unstimulated cells were used as a control group to reveal the stimulation effect. After stimulation, the cells were treated with sunitinib (1, 2, or 5 nM) or left untreated. Sunitinib was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). After a 24-hour stimulation, the cell proliferation was counted by visual cell count. Cell proliferation index was calculated according to proliferation results.

Smooth muscle cell migration was quantitated in Transwell culture chambers (Corning Inc., Corning, NY) where the up- per and lower culture chamber are separated by a polycar- bonate filter with 8-μm pores. The chambers were first coated with collagen (Rat Tail Collagen, Type 1, Sigma- Aldrich) at a concentration of 20 μg/mL at +4°C for 24 hours. Rat coronary artery SMC were seeded in the upper chamber. The PDGF-B was added to the lower chamber in Dulbecco's Modified Eagle Medium supplemented with 0.5% FCS and 0.1% BSA. The cells were treated with sunitinib (0.05, 0.1 or 0.2 μM) dissolved in dimethyl sulfoxide (Sigma) or left untreated. After 24 hours, the filters were removed, fixed with methanol, and stained with Mayers hematoxylin-eosin. Migrated cells (on the lower side of the filter) were quantitated by counting specified cross-sectional fields with a light micro- scope using 400 magnification. The in vitro experiment was repeated three times and was done in triplicates.

9. STATISTICAL ANALYSIS

The results are expressed as mean±SE. $P<0.05$ was accepted as significant. Significant differences between groups were determined by parametric analysis of variance and the least significant difference test, ANOVA, or Mann-Whitney non-

parametric analysis (SPSS, Chicago, IL). Serum creatinine levels between groups were analyzed by ANOVA for repeated measurements.

RESULTS

1. CLINICAL COURSE

All drugs studied were well tolerated. No adverse effects were observed and the overall condition of study animals and their behavior were normal in all study groups throughout the observation period (I-IV).

2. THE EFFECT OF ERLOTINIB AND SUNITINIB ON NEOINTIMAL FORMATION

Aortic denudation with an embolectomy catheter resulted in moderate asymmetric neointimal formation in the vehicle-treated group at day 14 (III and IV). Erlotinib ameliorated this neointima formation in a dose-dependent manner starting from 5 mg/kg/d (III). Sunitinib also had a dose-dependent effect on preventing neointima formation (IV). A significant reduction in the neointimal area was observed with a sunitinib dose of 1 mg/kg/d, and neointimal formation was almost completely prevented with 5 mg/kg/d and 20 mg/kg/d (IV).

3. THE EFFECT OF SUNITINIB ON SMC PROLIFERATION AND MIGRATION

PDGF-B is known to induce SMC proliferation in vitro and caused a 2.7-fold increase in SMC proliferation when compared to non-stimulated SMCs (IV). Sunitinib reduced SMC proliferation in a dose-dependent manner, and the highest concentrations completely prevented SMC proliferation (IV).

The PDGF-B induction caused a 2.8-fold SMC migration compared with unstimulated controls. This migration was dose-dependently decreased by sunitinib and the highest concentration prevented it completely (IV).

4. ACUTE REJECTION

In syngenic groups, no signs of acute rejection were observed at day 5, whereas monotherapy with CsA or Tac was characterized with moderate perivascular and diffuse interstitial inflammation (I, III, and IV). FK778 dose-dependently ameliorated early post-transplant inflammation in monotherapy (I). When FK778 was combined with CsA and Tac, it ameliorated perivascular and diffuse interstitial inflammation, indicating an additive effect on acute rejection (I). However, erlotinib (III) and sunitinib (IV) had no effects on the early inflammatory response at day 5.

In allografts of CsA and Tac-treated rats, significantly increased numbers of CD4+ and CD8+ lymphocytes and ED3+ activated macrophages were seen in the renal interstitium compared to syngenic DA-DA grafts at day 5 (I, III and IV).

FK778 monotherapy somewhat ameliorated the infiltration of both ED3+ activated macrophages and CD8+ lymphocytes, though no significant difference was found compared to CNI groups (I). The effect of FK778 monotherapy on CD4+ lymphocyte infiltration was not dose-dependent. The FK778 and CNI combination therapy decreased the amount of infiltrating CD8+ cells and activated macrophages at day 5, but the effect on CD4+ lymphocytes remained insignificant (I).

The TKIs erlotinib and sunitinib had no effect on the early post-transplant infiltration of CD4+ and CD8+ lymphocytes and ED3+ activated macrophages (III and IV).

5. GRAFT FUNCTION AND SERUM CREATININE LEVELS

Because the left-sided nephrectomy was performed on postoperative day 7 and serum sampling frequency was once per week, the serum creatinine level was a meaningful indicator of graft function only after week 2. Therefore, serum creatinine levels were not measured in the acute rejection study groups when they were sacrificed on day 5.

In the syngenic group, the post-transplant serum creatinine level was approximately 35 μmol/l (I-IV). In the CsA monotherapy group, an initial increase in the serum creatinine level was followed by a rather steady level of 60 μmol/l.

FK778 combined with CsA failed to decrease the creatinine levels at most time points (I). With the FK778-Tac combination, there was a trend towards lower creatinine levels compared to Tac monotherapy, though no significant difference was found between these groups (I).

TKIs had a significant effect on post-transplant graft function. Creatinine levels were significantly lower in imatinib-treated allografts than in CsA-monotherapy treated control allografts throughout the follow-up ($P<0.05$, II). In addition, when combined with CsA, erlotinib ($P=0.04$, III) and sunitinib ($P=0.02$, IV) significantly decreased the post-transplant serum creatinine level, maintaining better graft function.

6. CHRONIC REJECTION

No histological signs of chronic rejection were observed in syngenic grafts 90 days after transplantation, whereas moderate chronic changes (e.g., increased fibrosis, inflammation, and intimal proliferation) were observed in allografts treated with CsA (I-IV) or Tac monotherapy (I). The combination of FK778 and CNI significantly ameliorated the inflammation, fibrosis, and intimal proliferation observed in CNI monotherapy-treated allografts (I).

Short-term imatinib treatment (combination therapy with CsA) significantly ameliorated the development of chronic rejection compared to control allografts (II), and only mild changes were observed. Interstitial inflammation and fibrosis, as well as arterial intimal proliferation and increased glomerular mesangial matrix, were significantly decreased in imatinib-treated allografts. In addition, the infiltration of

CD4+ and CD8+ T cells and activated macrophages was decreased (II).

Erlotinib significantly ameliorated both the CADI score and individual CADI parameters (interstitial inflammation and mesangial matrix increase, P=0.02; fibrosis, P=0.03; intimal proliferation, P=0.04), CD4+ and CD8+ T-cell (P=0.04) and B-cell infiltration on day 90 (P=0.01, III).

Sunitinib decreased the CADI score when combined with CsA. Sunitinib also significantly ameliorated chronic inflammation, fibrosis, increase in mesangial matrix, and intimal proliferation, as well as the infiltration of CD4+ and CD8+ lymphocytes and ED3+ activated macrophages (IV).

7. POST-TRANSPLANT GROWTH FACTOR EXPRESSION

7.1 PDGF

In syngenic controls, PGDF expression (PDGF-A, PDGF-B, PDGFR-α, PDGFR-β) was almost non-existent 5 and 90 days after transplantation (I, II, and IV). In the CsA and Tac groups, PDGF expression was significantly increased by day 5, and this expression remained elevated at day 90 (I, II, and IV). PDGF ligand and receptor expression was noted in capillary endothelial cells and infiltrating leukocytes (I, II, and IV). The difference in PDGF ligand and receptor expression between CsA and Tac monotherapy groups remained relatively small (I). When FK778 was combined with CNIs, it significantly decreased the expression of PDGF ligands and receptors, both acute and chronic (I).

Short-term imatinib treatment significantly inhibited the induction of PDGF-A and -B ligands and PDGF receptor –β (II). Expression of both PDGF-A and -B was decreased, especially in interstitial leukocytes and tubules (II). PDGF-B expression

was also significantly inhibited in arterial SMCs in imatinib-treated allografts compared to control allografts (II). The expression of PDGFR-β was decreased markedly in graft-infiltrating inflammatory cells, tubular area and in arterial SMCs (II).

Sunitinib significantly decreased acute arterial expression of PDGFR-β at day 5 but failed to affect acute PDGF-A, PDGF-B or PDGFR-α expression (IV). However, sunitinib significantly decreased chronic PGDF expression (IV).

7.2 TGF-β

TGF-β expression was low in syngenic grafts at both 5 and 90 days (I). However, moderate TGF-β and TGF-βR expression was observed in the CsA and Tac groups at day 5, and this expression was mainly localized in the renal capillaries and infiltrating leukocytes (I). This expression remained elevated at day 90 in the CsA group, but only mild expression was observed in the Tac monotherapy-treated allografts (I).

When FK778 was combined with CNIs, it significantly decreased the expression of TGF-β and TGF-βR, both acute and chronic, compared to CNI monotherapy (I). In addition, short-term imatinib treatment significantly inhibited the induction of TGF-β and TGF-βR in inflammatory cells and TGF-βR induction also in tubules at day 90 (II).

7.3 VEGF

No pathological VEGF expression was observed in syngenic grafts at day 5 or 90 (IV). However, significantly increased tubular, arterial, and inflammatory expression of VEGF-A was observed in CsA-treated allografts at day 5 and 90 (IV). VEGF-B

was expressed mainly in the arterial and tubular area and in inflammatory cells. The expression pattern of VEGFR-1 and -2 was somewhat broader than that of VEGF ligands and also glomerular expression was observed (IV).

Sunitinib significantly decreased acute arterial expression of VEGF-A and VEGF-B at day 5 (IV). In addition, prolonged VEGF ligand and receptor expression was prevented with sunitinib treatment (IV).

7.4 EGF

No expression of EGF or EGFR was observed in syngenic grafts at day 5 or 90 (III). However, the expression of EGF and EGFR was significantly increased in CsA-treated allografts at day 5, and this expression remained elevated at day 90 (III). The increased EGF expression was mainly localized to inflammatory cells, whereas increased EGFR expression was observed in inflammatory cells and tubular cells.

Erlotinib had no effect on acute EGF or EGFR expression (III). However, it significantly ameliorated chronic EGF expression ($P=0.03$; III). Erlotinib also significantly ameliorated chronic EGFR expression in inflammatory cells and tubular cells (III).

DISCUSSION

1. GROWTH FACTOR EXPRESSION IN SYNGENIC GRAFTS

Expression of PDGF-A and -B, PDGFR-α, PDGFR-β, TGF-β, TGF-βR, EGF, EGFR, VEGF-A, VEGF-B, VEGFR-1, and VEGFR-2 was low in syngenic kidney grafts in all studies and at all time points (I-IV). This indicates that ischemia reperfusion injury or surgery-related factors alone are not able to induce prolonged growth factor expression in this animal model. This also suggests that innate immunity alone, without allorecognition, does not lead to allograft injury.

2. FK778 AMELIORATES ACUTE AND CHRONIC REJECTION

According to the results of article I, FK778 decreases acute rejection in a dose-dependent manner. Because FK778 is an immunosuppressive drug, this effect is only logical. However, FK778 monotherapy also decreased the expression of PDGF and TGF-β ligands and receptors in a dose-dependent manner. Therefore, FK778 appears to have a beneficial effect on growth factor expression, unlike CsA (Iacopino et al. 1997) and Tac (Khanna, Cairns, Hosenpud 1999). This effect on TGF-β and PDGF ligands and receptors remained when FK778 was combined with CsA or Tac. Thus, FK778 appears to have additive effects with CNIs with regard to the effect on the early post-transplant inflammatory response and favorable effects on growth factor expression. Therefore, FK778 could be used in combination therapy with both CsA and Tac for acute rejection.

FFK778 also significantly decreased the chronic inflammation, fibrosis, and intimal proliferation seen in CNI monotherapy-treated allografts (I). This effect may be due, in part, to decreased growth factor expression. Unfortunately, clinical studies with

FK778 were discontinued when no results were seen for the rate of acute rejection in renal transplant patients after 1 year (Wlodarczyk et al. 2012). However, acute rejection in clinical kidney transplantation may already be treated successfully with current multi-therapy of CNI, corticosteroids and anti-thymocyte globulin, whereas chronic rejection remains the main reason for late allograft loss. According to the results of study I, FK778 could have had beneficial effects on chronic rejection in clinical transplantation if it had been launched for clinical use in transplant patients.

3. SHORT-TERM IMATINIB TREATMENT PREVENTS CHRONIC REJECTION

Imatinib inhibits the PDGF response and could be a potential intervention in clinical kidney transplantation (Savikko et. al. 2003). However, growth factors also have physiological effects, and their prolonged inhibition could cause adverse effects. Life-long daily administration would also be very expensive.

The results of article II show that even short-term imatinib treatment is sufficient for preventing the development of chronic allograft injury, and that it preserves the long-term graft function. Early short-term treatment with imatinib also decreases chronic PDGF and TGF-β expression, and growth factor expression remains low even after imatinib has been discontinued. This indicates that the early induction of PDGF and TGF-β is an important mechanism in the pathophysiology of chronic rejection. Therefore, early short-term inhibition of the growth factor response could prevent the response altogether, and TKIs could be considered for induction therapies in clinical transplantation.

According to these results, life-long TKI therapy may not be necessary for the prevention of chronic allograft injury. This makes TKIs a more convenient option for clinical kidney transplantation.

4. EGF INHIBITION WITH ERLOTINIB PREVENTS CHRONIC REJECTION AND MAINTAINS RENAL FUNCTION

Erlotinib is a highly selective EGF-inhibiting TKI, and the role of EGF in the development of acute and chronic rejection has not been fully investigated. Chronic rejection is partly driven by arterial intimal proliferation, and growth factors participate in this process resembling of accelerated atherosclerosis. According to the results of paper III, inhibition of EGF by erlotinib decreases intimal proliferation after aortic denudation, suggesting that erlotinib may have beneficial effects on allograft vasculopathy.

EGF and EGFR expression was increased during acute rejection. Inhibition of EGF by erlotinib had no effect on this early post-transplant expression when combined with CsA. However, erlotinib decreased chronic inflammation, fibrosis, the increase in mesangial matrix, and intimal proliferation. Therefore, inhibition of EGF by erlotinib prevents histological changes associated with rejection in kidney allografts. In addition, chronic EGF and EGFR expression decreased, and only marginal expression was observed, suggesting an absence of active fibrotic processes. Based on lower serum creatinine levels, erlotinib also maintains better allograft function than CsA monotherapy. This is an important finding because serum creatinine levels are an insensitive marker of kidney function, and a pronounced effect is needed before creatinine levels change.

Erlotinib is not an immunosuppressive drug; it has no direct effect on acute rejection. This is important because it emphasizes that the prevention of changes associated with chronic rejection here (III) was not achieved by increased immunosuppression or decreased acute rejection, a known risk factor for chronic rejection. Erlotinib inhibits

the EGF response by inhibiting the EGF signaling cascade, affecting downstream from early EGF and EGFR expression that was monitored on day 5 by immunohistochemistry. Therefore, erlotinib has no effect on the amount of EGF or EGFR; instead, erlotinib inhibits the signaling cascade these ligands and receptors mediate.

Taken together, these results indicate that EGF plays a profound role in the development of chronic rejection, and erlotinib could have a beneficial effect on chronic rejection in clinical transplantation.

5. SUNITINIB PREVENTS CHRONIC ALLOGRAFT INJURY AND PRESERVES KIDNEY FUNCTION

Sunitinib is a potent TKI with a wide range of activity and a more potent effect on PDGF inhibition than imatinib. Therefore, sunitinib could be an efficient intervention in chronic rejection. According to the results of article IV, sunitinib prevents neointimal formation after aortic denudation and decreases neointimal PDGF expression. This finding indicates that sunitinib has beneficial effects on post-transplant vasculopathy. This effect may be mediated, in part, by the prevention of SMC proliferation and migration. Similar to erlotinib, sunitinib has no specific immunosuppressive mechanism and no effect on the early inflammatory response and acute rejection. However, as sunitinib is a potent inhibitor of both PDGF and VEGF, it decreased the early arterial expression of PDGFR-β, VEGF-A, VEGF-B, VEGFR-1, and VEGFR-2. This may mediate its effect on chronic rejection. Sunitinib, like erlotinib and imatinib, exerts its effects by binding to the intracellular ATP-binding site of tyrosine kinases (Kim et al. 2006), inhibiting the function of growth factors instead of their early post-transplant expression. This may partly explain why

sunitinib had no effect on intense inflammatory PDGF and VEGF expression but profoundly affected later chronic rejection changes.

Sunitinib significantly decreased the changes associated with chronic rejection when combined with CsA and resulted in nearly normal histology at day 90 (IV). In addition, chronic PDGF and VEGF expression was almost completely prevented and, according to serum creatinine levels, sunitinib maintained better allograft function. These results indicate that combined inhibition of PDGF and VEGF by sunitinib effectively decreases chronic allograft injury in an experimental kidney transplantation model. Therefore, sunitinib could have beneficial effects for the prevention of chronic rejection in clinical kidney transplantation.

6. TYROSINE KINASE INHIBITION IN EXPERIMENTAL KIDNEY TRANSPLANTATION

Growth factors are induced in various steps along the progression of disease in kidney transplant patients. Growth factor expression increases during the development of the kidney disease that originally leads to ESRD and the need for kidney transplantation, but may also be activated during the donor brain death and transplantation operation itself. In addition, growth factors are induced by innate immunity and ischemia reperfusion injury, which may in turn strengthen the acute rejection, a major risk factor for chronic rejection.

Our studies found significant expression of PDGF, VEGF, TGF-β, and EGF during acute rejection in experimental kidney transplantation. Their inhibition with orally administered TKIs decreased the proliferation and migration of SMCs, as well as experimental allograft vasculopathy. Furthermore, inhibition of growth factor receptors decreased chronic rejection changes via a mechanism that differs from

cellular infiltration and appears to be additive with that of CNIs. Therefore, several TKIs shown here profoundly decreased chronic rejection changes, suggesting a class effect that could achieve beneficial results for kidney transplant patients.

In clinical oncology tyrosine kinase inhibitors are being concidered to be used also in combination therapies regarding individual genetic factors and thus allowing personalized therapy for cancer patients (Broekman et al. 2011). The efficasy of growth factor inhibition or different TKIs against individual cancer depends on spesific mutations causing the cancer which may explain why TKIs differ in their effect. However, in kidney transplantation the immune response is not dependent on patient spesific mutations. Therefore one can speculate that TKI-treatment could be more standardized in clinical transplantation than in oncological field and also combination of different TKIs could be studied.

Our results in article II suggest that life long growth factor inhibition may not be mandatory to prevent the development of chronic rejection changes. The length of a study periode with a one month induction therapy in experimental model is hard to extrapolate into clinical transplantation. However, results presented here indicate that the early post-transplant events and growth factor responses are important in the development of chronic rejection. The differences between different TKIs in the prevention of these changes can not be answered based on our sudies because they were not compared between each other. However, because of the strong correlation of results with these four different tyrosine kinase inhibiting drugs on the prevention of chronic rejection, we propose that tyrosine kinase inhibition should be studied also in clinical transplantation.

CONCLUSIONS

FK778 inhibits several growth factor RTKs and offers a different immunosuppressive mechanism that could supplement the effect of CNIs. Therefore, FK778 could be a valuable option for combination therapy with CNIs. Unfortunately, the clinical studies with FK778 were stopped when no significant benefit was observed in acute rejection after 1 year of follow-up, though the side effect profile was milder with FK778 than other immunosuppressive agents (Wlodarczyk et al. 2012). Our results in experimental model (I) indicate that FK778 could be a potent intervention for preventing chronic rejection. However, the effect of FK778 on chronic rejection in clinical kidney transplantation cannot be evaluated according to the 1-year follow-up results of transplantation patients. Thus, the potential effect of FK778 on chronic rejection remains unknown.

Our results in article II on short-term imatinib treatment demonstrate that early PDGF induction plays an important role in the cellular processes leading to chronic rejection. If this early PDGF induction is prevented with the combination of imatinib and CsA, the fibrotic cascade leading to chronic allograft injury may be prevented. Thus, early PDGF inhibition with imatinib could be used as an induction therapy in clinical kidney transplantation.

We showed in article III that EGF and EGFR expression is increased in rejecting renal allografts after kidney transplantation. EGF inhibitor erlotinib decreases late post-transplant EGF and EGFR expression when combined with CsA and prevents changes associated with chronic rejection. Furthermore, erlotinib maintains better allograft function after experimental kidney transplantation. This effect is not achieved by decreased acute rejection indicating that EGF is significantly involved in the pathogenesis of chronic allograft injury. Thus, inhibition of EGF by erlotinib

could improve renal allograft outcomes in kidney transplant patients.

According to article IV, sunitinib prevents chronic rejection in an experimental rat kidney transplantation model when used in combination therapy with CsA. Also, renal graft function was preserved better with sunitinib treatment than CsA monotherapy. These results indicate that sunitinib could have beneficial effects in clinical transplantation.

Taken together, the results in this project show that chronic rejection may be prevented by the inhibition of PDGF and TGF-b, PDGF and VEGF, or EGF. With a single TKI, it is not possible to completely rule out the existence of multiple pathways by other growth factors behind this effect, especially as growth factors have multiple interactions. However, these results indicate that growth factors play a significant role in mediating the rejection process, and this may be treated with TKIs, which are already used clinically and could offer a new intervention against allograft rejection.

LIMITATIONS OF THE STUDY

We used a standardized rat model of experimental kidney transplantation. This model is immunologically restricted and enables the investigation of the immunological rejection process. Our rats were healthy, meaning that they had no background disease resulting in ESRD and a need for kidney transplantation. This affects our results because, during the experimental transplantation, the recipients are not uremic and have no background disease that could affect renal function or the immunological response in the post-transplant period. Therefore, our model differs from clinical kidney transplantation, where even more pathological processes participate in renal injury. However, a functional kidney transplantation model with 300-g rats is challenging, even with healthy rats. We recognize that our animal model is not

perfect, but to the best of our knowledge, an experimental transplantation model with uremic rats does not exist.

The number of animals we were aiming for in each group during this project was 5, which is a compromise between reliable results and minimal usage of laboratory animals. We recognize that this could be regarded as a small group size. However, keeping in mind the hundreds of kidney transplantations performed for this project, we can assure that requiring a bigger group size is much easier than providing it.

The publications leaned heavily on immunohistochemistry, and background staining remains a problem when staining renal growth factors in rat. Rejecting samples may also be distinguishable in blind scoring due to their histological appearance, and this may cause bias. However, growth factors have been investigated in various acute and chronic kidney models and the vast literature supports our results that growth factors are increased during kidney disease and the rejection process. In addition, growth factor inhibition with TKIs significantly decreased histological rejection, indicating that growth factors play a profound role in the rejection process. TKIs also decreased serum creatinine levels, which is the most important and objective finding supporting the efficacy of these drugs in chronic rejection.

SUMMARY

Chronic allograft injury is a multifactorial process mediated by both immunological and non-immunological factors, leading to organ dysfunction and humoral and cellular rejection. Currently, no specific treatment is available for chronic allograft injury. Pathological growth factor expression is observed before the development of end-stage renal disease (ESRD), and several growth factors (e.g., PDGF, TGF-β, EGF, and VEGF) are induced during the rejection process. This increased growth factor expression is induced by multiple pathways that are unaffected or partly induced by modern immunosuppressive medication. Individual growth factors induce their own expression and, via interactions, the expression of other growth factors. This cascade may lead to prolonged growth factor expression and subsequent chronic allograft injury.

Many growth factors exert their effects through specific receptor tyrosine kinases (RTKs). RTKs can be inhibited by novel tyrosine kinase inhibitors (TKIs), which are orally administered and clinically used to treat several malignancies. RTK inhibition is achieved by competitive inhibition of intracellular ATP binding. In our experiments, we tested the efficacy of four different drugs with RTK-inhibition activity on chronic rejection in a rat transplantation model.

First, we investigated FK778, an analogue of the active metabolite of leflunomide, which inhibits T- and B-cell proliferation and functions by interfering in de novo pyrimidine biosynthesis. FK778 also inhibits PDGF-RTK both in vitro and in vivo. In our experiments, FK778 decreased acute rejection in a dose-dependent manner and had additive effects with both cyclosporine and tacrolimus, decreasing both acute and chronic rejection. FK778 also decreased post-transplant PDGF and TGF-β expression.

In our second study, we investigated short-term imatinib treatment in a chronic rejection model. According to our results, short-term (i.e., 30-day) treatment with PDGF-inhibiting TKI imatinib is sufficient to decrease subsequent chronic rejection changes at day 90. This short-term imatinib treatment also decreased post-transplant PDGF and TGF-β expression and restored allograft function.

In the third study, we used erlotinib, a specific EGF-inhibiting TKI. We showed that, when combined with cyclosporine, erlotinib prevents chronic rejection changes and maintains better graft renal function. Erlotinib also decreased post-transplant EGF expression.

In our fourth study we tested a potent, PDGF- and VEGF-inhibiting TKI, sunitinib, in combination with cyclosporine. Our data showed that sunitinib decreased early arterial expression of VEGF and PDGF as well as chronic expression of both PDGF and VEGF. Sunitinib decreased chronic rejection and maintained better graft function after transplantation.

Taken together, our results show that growth factor inhibition via the inhibition of tyrosine kinases is a potent pathway to prevent chronic rejection and maintain graft function in the transplanted kidney. Our finding that even short-term imatinib treatment is sufficient to prevent chronic rejection indicates that life-long treatment may not be mandatory and early post-transplant events are crucial in mediating chronic rejection changes. TKIs imatinib, erlotinib, and sunitinib are well tolerated when combined with cyclosporine and prevent chronic rejection in an experimental kidney transplantation model. In addition, these drugs maintain better graft function after transplantation. Therefore, they could have beneficial effects in clinical transplantation.

YHTEENVETO (FINNISH SUMMARY)

Munuaissiirto on hoitotuloksiltaan loppuvaiheen munuaisen vajaatoiminnan paras hoitomuoto sekä pitkäaikaisennusteen että elämänlaadun kannalta. Krooninen hyljintäreaktio on kuitenkin edelleen merkittävä munuaissiirtojen pitkäaikaistuloksia heikentävä tekijä eikä siihen tunneta toimivaa hoitoa. Kroonisen hyljinnän kehittymiseen vaikuttavat monet sekä immunologiset että immunologiasta riippumattomat tekijät ja myös potilaan edeltävä munuaissairaus voi vaikuttaa hyljintäreaktion voimakkuuteen.

Monet kasvutekijät, kuten verihiutalekasvutekijä PDGF, transformoiva kasvutekijä TGF-beta, verisuonikasvutekijä VEGF ja epidermaalinen kasvutekijä EGF vaikuttavat jo loppuvaiheen munuaisen vajaatoiminnassa munuaisen fibrotisoitumiseen ja sitä kautta munuaisen toimminnan hiipumiseen. Lisäksi näiden kasvutekijöiden ilmentyminen on lisääntynyt hyljintäreaktion aikana eikä nykyisin käytössä olevalla immunosupressiivisella lääkityksellä pystytä estämään kasvutekijäreaktion aktivoitumista munuaissiirteessä.

Monet kasvutekijät välittävät vaikutuksensa kohdesolun pinnalla olevien tyrosiinikinaasi-entsyymeinä toimivien reseptoreidensa kautta. Koska krooninen kasvutekijöiden yli-ilmentyminen on tyypillistä pahanlaatuisten kasvaimien yhteydessä, on näitä reseptoreita estämään kehitetty useita onkologisia lääkkeitä. Väitöstutkimuksen tarkoituksena oli selvittää neljän, eri tavalla tyrosiinikinaasi-entsyymejä estävän, lääkeaineen tehoa munuaissiirteen kroonisen hyljinnän estossa kokeellisessa rotan munuaistensiirtomallissa.

Ensimmäisessä osatyössä selvitettiin kokeellisen immunosupressiivisen lääkeaineen FK778:n tehoa yhdistettynä nykyisin käytössä oleviin kalsineuriiniestäjiin siklosporiiniin ja takrolimuusiin. Verihiutalekasvutekijä PDGF:n reseptoria estävä

FK778 esti sekä akuutin, että kroonisen hyljintäreaktion kehittymistä yhdistelmäterapiana kalsineuriiniestäjien kanssa ja lisäksi verihiutalekasvutekijä PDGF:n ja transformoiva kasvutekijä TFG-betan ilmentyminen munuaissiirteessä väheni.

Toisessa osatyössä tutkittiin lyhytkestoisen imatinibihoidon tehoa kroonisen hyljinnän estossa yhdistettynä siklosporiiniin. Imatinibi on tyrosiinikinaasiestäjä, joka estää verihiutalekasvutekijän reseptoria ja siten PDGF:n vaikutusta. Tulosten perusteella alkuvaiheen imatinibihoito vähentää PDGF:n ja TGF-betan ilmentymistä munuaissiirteessä ja estää kroonisen hyljintäreaktion kehittymisen. Lisäksi alkuvaiheen imatinibihoito paransi siirteen munuaisfunktiota ja tämä vaikutus jatkui myös imatinibihoidon lopettamisen jälkeen. Näin ollen induktiotyyppinen alkuvaiheen kasvutekijäesto tyrosiinikinaasiestäjällä voisi estää myös myöhemmän hyljintäreaktion kehittymisen eikä elinikäinen lääkitys olisi välttämätöntä.

Kolmannessa osatyössä selvitettiin epidermaalikasvutekijä EGF:n reseptoria estävän erlotinibin tehoa kroonisen hyljinnän estossa yhdistämällä sitä siklosporiinilääkitykseen. Tulosten perusteella erlotinibi estää kroonisen hyljinnän kehittymistä ja parantaa siirteen munuaisfunktiota pelkkään siklosporiinilääkitykseen verrattuna. Tulosten perusteella epidermaalikasvutekijä osallistuu kroonisen hyljinnän kehittymiseen munuaissiirteessä.

Neljännessä osatyössä tutkittiin sekä verisuonikasvutekijä VEGF:ä että verihiutalekasvutekijä PDGF:ä estävää sunitinibia kroonisen hyljinnän estossa yhdessä siklosporiinin kanssa. Tulosten perusteella PDGF:n ja VEGF:n samanaikainen esto on tehokas hoito kroonisen hyljinnän estämisessä kokeellisessa munuaissiirtomallissa. Lisäksi myös sunitinibi paransi siirteen munuaisfunktiota pelkkään siklosporiinilääkitykseen verrattuna.

Tutkimustulostemme perusteella kasvutekijöiden yli-ilmentyminen on merkittävä kroonisen hyljinnän kehittymiseen liittyvä tekijä. Jo nyt kliinisessä käytössä olevillä tyrosiinikinaasiestäjillä imatinibilla, erlotinibilla ja sunitinibilla voidaan tehokkaasti estää kasvutekijöiden yli-ilmentyminen munuaissiirteessä ja kokeellisessa munuaissiirtomallissa tämä ennaltaehkäisee kroonisen hyljintäreaktion kehittymisen lähes täydellisesti. Lisäksi kasvutekijäilmentymisen farmakologinen estäminen tyrosiinikinaasiestäjillä parantaa siirteen munuaisfunktiota pelkkään siklosporiinilääkitykseen verrattuna. Tulostemme perusteella lyhytaikainen munuaissiirron jälkeinen hoito saattaisi estää myös myöhempää hyljintäreaktion kehittymistä eikä elinikäinen tyrosiinikinaasiestäjähoito olisi välttämätöntä. Näin ollen kasvutekijävaikutuksen estäminen tyrosiinikinaasiestäjillä saattaisi olla tehokas hoitomuoto myös kliinisessä munuaissiirtotoiminnassa.

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